SPECIFICATION

PROSTACYCLIN SYNTHASE DERIVED FROM HUMAN

Technical Field

The present invention relates to a polypeptide having an amino acid sequence of human-originated prostacyclin synthase (hereinafter referred to as PGIS), a DNA encoding same, a vector containing said DNA, a host cell transformed with said vector and a method for preparing human-originated PGIS comprising culturing said host cell. The present invention also relates to an antibody having a reactivity with said PGIS or its fragment. Moreover, the present invention relates to a pharmaceutical composition comprising said DNA or a vector containing said DNA, a method for promoting the production of prostaglandin I_2 and a method for the treatment of the diseases induced by a low production of prostaglandin I_2.

Background Art

PGIS is mainly contained in microsomal fractions of vascular endothelial cells, and is an enzyme that catalyzes synthesis of prostaglandin I_2 (hereinafter referred to as PGI_2), that is, conversion of prostaglandin H_2 (hereinafter referred to as PGH_2) to PGI_2.

PGI_2 synthesized by this enzyme has potent platelet aggregation-inhibitory action and vascular smooth muscle-relaxing action. On the other hand, platelets contain thromboxane A_2 (hereinafter referred to as TXA_2) having strong platelet aggregation action and vascular smooth muscle-contracting action, and the both substances act antagonistically in the vascular system to maintain homeostasis [British Journal of Pharmacology, vol. 76, p 3 (1982)].

Cardiovascular diseases such as myocardial infarction, thrombosis and arteriosclerosis, which are among the adult diseases, have recently been considered to be caused by the imbalance in the vascular production of PGI_2 and TXA_2, particularly, insufficient vascular function due to low production of PGI_2 (ibid.).

For the therapeutic treatment of the diseases presumably induced

by the low production of PGI_2, PGI_2 may be supplemented as a pharmaceutical product from the outside of the body. However, PGI_2 is chemically extremely unstable to the extent that a practical use of PGI_2 itself as a pharmaceutical product may be unrealizable. In view of such situation, for example, stable PGI_2 analogs such as blood coagulation inhibitor or vasodilator are under development.

The homeostasis in human and other animals which is inherently based on the balance between PGI_2 and TXA_2 may possibly destroyed by the administration of stable PGI_2 analogs. That is, administration of stable PGI_2 analogs in large amounts is associated with a risk of lowering the responsiveness of cells to PGI_2, thus impairing its capability of responding to PGI_2 when such responsiveness is in urgent need [Prostaglandins, vol. 19, p 2 (1980)].

For correcting the imbalance between PGI_2 and TXA_2 and attempting the recovery of normal functions of the vascular system in an expectation of therapeutic effect over thrombosis and the like, chemically stable analogs may be used. Alongside therewith, moreover, elucidation of physicochemical property and biological property of PGIS, clarification of the relations between PGIS production and PGI_2 production while using said PGIS or DNA encoding PGIS as a research sample, and development of said PGIS or DNA encoding PGIS as pharmaceutical products to regulate the production of PGI_2 are considered to be important and significant for the treatment of the above-said various diseases caused by the imbalance between PGI_2 and TXA_2.

Conventionally, there has been reported the tissue distribution of PGIS, namely, its presence in vascular endothelial cells, non-vascular smooth muscle cells and arterial smooth muscle of various organs [Advances in Prostaglandin, Thromboxane, and Leukotriene Research, vol. 11, pp. 87-92 (1983) and J. Biol. Chem., vol. 258, No. 9, pp. 5922-5926 (1983)]. Meanwhile, isolation and purification of PGIS from porcine and bovine have been tried [porcine: Cytochrome P450, Biochemistry, Biophysics and Environmental Implications, pp.

103-106 (1982); bovine: J. Biol. Chem., vol. 258, No. 9, pp. 3285-3293 (1983)] and N-terminal amino acid sequence and partial downstream amino acid sequence of bovine PGIS have been reported [Advances in Prostaglandin, Thromboxane, and Leukotriene Research, vol. 17, pp. 29-33 (1987) and Biochemical and Biophysical Research Communications, vol. 197, No. 3, pp. 1041-1048 (1993)].

However, isolation, purification and amino acid sequence of human PGIS have not been elucidated.

Disclosure of the Invention

An object of the present invention is to clarify an amino acid sequence of PGIS derived from human and provide said human-originated PGIS and DNA encoding said PGIS.

Said PGIS and DNA encoding said PGIS are useful as reagents for ① the analysis of the physicochemical and biological properties of PGIS at the molecular or genetic level; ② the analysis of the mechanism controlling the production of PGIS and the mechanism controlling the production of PGI_2 by PGIS; and ③ the investigation of the cause of various cardiovascular diseases considered to be induced by the production imbalance between PGI_2 and TXA_2, and the molecular or genetic level analysis for the development of therapeutic agents for said diseases. In addition, PGIS and its mRNA are useful as diagnostics for the determination of expression level and distribution in the body tissues. Still further, they are expected to provide therapeutic agents for, for example, various cardiovascular disorders such as thrombosis, myocardial infarction, arteriosclerosis and angina pectoris, which enhance the production level of PGI_2 upon introduction of themselves, fragment thereof or modified compound thereof into the body in a lesion-specific manner.

Another object of the present invention is to provide a recombinant vector containing a DNA encoding human-originated PGIS, the expression system of PGIS which comprises a host cell transformed with said vector, and a method for preparing PGIS by genetic engineering using said expression system.

According to such method, human-originated PGIS can be prepared in great amounts with ease and with high efficiency.

The present invention also aims at providing a human-originated PGIS antibody useful for the purification of human-originated PGIS and immunohistochemical analysis of the cause of a disease.

The present inventor has conducted intensive studies with the aim of accomplishing the above-mentioned objects, and succeeded in cloning cDNA encoding PGIS from human aorta endothelial cells and identifying the primary structure of human-originated PGIS from the nucleotide sequence of said cDNA, which resulted in the completion of the present invention.

Accordingly, the present invention relates to a DNA comprising a DNA having a nucleotide sequence encoding an amino acid sequence of human-originated PGIS substantially depicted in Sequence No. 12, preferably a DNA comprising a DNA having a 28th-1527th nucleotide sequence substantially shown in Sequence No. 11, and more preferably a DNA having a 28th-1527th nucleotide sequence shown in Sequence No. 11.

The present invention also relates to a recombinant vector comprising the above-mentioned DNA, a host cell transformed with said vector and a method for preparing human-originated PGIS comprising culturing said host cell in a medium and recovering human-originated PGIS from the obtained culture.

The present invention also relates to a polypeptide having an amino acid sequence of human-originated PGIS which is substantially shown in Sequence No. 12, and antibodies having reactivities with said human-originated PGIS.

The present invention further relates to a pharmaceutical composition comprising said DNA or a recombinant vector comprising said DNA. Said pharmaceutical composition can be used as a medicament for promoting PGI_2 production or for treating the diseases induced by a low production of PGI_2.

The present invention moreover relates to a method for promoting

the production of PGI_2, comprising introducing the above-mentioned DNA or a recombinant vector comprising said DNA into human or other animals. The present invention also relates to a method for treating the diseases induced by a low production of PGI_2, comprising introducing the above-mentioned DNA or a recombinant vector comprising said DNA into human or other animals.

Brief Description of the Drawings

Fig. 1 shows a restriction enzyme map of human PGIS cDNA, and the PGIS DNA region comprised in λhPGIS141, pHPGIS135 and pHPGIS36.

Fig. 2 shows a restriction enzyme map of plasmid pHPGIS36.

Fig. 3 shows a restriction enzyme map of plasmid pHPGIS135.

Fig. 4 shows a restriction enzyme map of plasmid pHPGIS1.

Fig. 5 shows a restriction enzyme map of human PGIS expression vector pCMV-HPGIS1.

Fig. 6 shows an expression vector pUC-CAGGS.

Fig. 7 is a photograph showing the results of the analysis, by thin layer chromatography, of the PGIS activity in the cells into which pCMV-HPGIS1 has been introduced.

Fig. 8 is a photograph showing the results of the analysis, by thin layer chromatography, of the PGIS activity in positive control (bovine platelet microsomes).

Fig. 9 is a photograph showing the results of the analysis, by thin layer chromatography, of negative control wherein pCMV alone was introduced.

Fig. 10 is a graph showing the effects of the introduction of human PGIS expression vector on the blood vessel smooth muscle cell proliferation.

Fig. 11 is a photograph showing the results of RNA blot (electrophoresis) analysis of human pGIS mRNA treated with cytokines.

Fig. 12 is a photograph showing the distribution of PGIS mRNA expression in human body (pancreas, kidney, skeletal muscle, liver, lung, placenta, brain and heart) by electrophoresis.

Fig. 13 is a photograph showing the distribution of PGIS mRNA

expression in human body (peripheral leukocyte, large intestine, small intestine, ovary, testicle, prostate, thymus and spleen) by electrophoresis.

Detailed Description of the Invention

The present invention is explained in detail in the following.

The polypeptide of the present invention has a catalytic activity to convert PGH_2 to PGI_2 and has an amino acid sequence of human-originated PGIS substantially shown in Sequence Listing, Sequence No. 12 to be mentioned later.

By "substantially" is meant that the polypeptide of the present invention is not limited to the polypeptide having the amino acid sequence shown in Sequence No. 12, but may include deletion, substitution and addition with respect to some of the amino acids in the amino acid sequence shown in Sequence No. 12, as long as the polypeptide has immunological and biological activity (human PGIS activity) similar to that of human-originated PGIS having said amino acid sequence.

While the site of deletion, substitution and addition of the amino acids is not particularly limited, at least 441st Cys residue and thereabout region in the amino acid sequence shown in Sequence No. 12 need to be reserved. This is because human-originated PGIS of the present invention is homologous to known cytochrome P450 in the amino acid sequence, since it has Cys residue in the C-terminal side of the amino acid sequence constituting the heme-binding site (fifth ligand) which is important for the expression of biological activity of cytochrome P450, and speculated to be a new protein belonging to the cytochrome P450 family [see *Seibutsu Butsuri*, vol. 32, No. 1, pp. 10-15 (1992)].

The polypeptide of the present invention preferably has an amino acid sequence of human-originated PGIS shown in Sequence No. 12.

The PGIS activity possessed by the polypeptide of the present invention is a catalytic activity to convert PGH_2 to PGI_2. Said PGIS activity can be determined according to the method of Salmon, J.A.

and Flower, R.J. et al [Methods Enzymol., *86*, pp. 91-99 (1982)] wherein the conversion of ^{14}C-labeled PGH_2 to PGI_2 is assayed by separating the metabolite of 6-keto-$PGF_{1\alpha}$ by thin layer chromatography and detecting the radioactivity of said 6-keto-$PGF_{1\alpha}$.

The present invention also relates to a DNA comprising a DNA having a nucleotide sequence encoding the amino acid sequence of human-originated PGIS substantially shown in Sequence No. 12.

Said DNA may be any as long as it comprises a DNA having a nucleotide sequence encoding the aforementioned amino acid sequence of human-originated PGIS, and is exemplified by a DNA encoding the polypeptide having the amino acid sequence shown in Sequence No. 12 or a polypeptide having the equivalent immunological and biological activity. More specifically, it is a DNA comprising the 28th-1572nd nucleotide sequence in the nucleotide sequence shown in Sequence No. 11.

In general terms, the genetic recombinant technique enables conversion of at least one nucleotide of a DNA sequence of a gene to a different nucleotide according to the degeneracy of the genetic code, without changing the amino acid sequence of a protein produced by the gene. Accordingly, the DNA of the present invention encompasses a DNA comprising a nucleotide sequence obtained by modification for substitution, based on the genetic code, of the 28th-1527th nucleotide sequence of Sequence Listing Sequence No. 11.

The DNA of the present invention can be obtained by any method. For example, the present invention encompasses complementary DNA (cDNA) prepared from mRNA, DNA prepared from genomic DNA, DNA obtained by chemical synthesis, DNA obtained by amplification by PCR using RNA or DNA as a template, and DNA constructed by suitably combining these methods.

The DNA of the present invention can be obtained by a method comprising cloning cDNA from mRNA of human-originated PGIS by a conventional method, a method comprising splicing an isolated genomic DNA for PGIS, a method comprising chemical synthesis or other method.

(1) For example, a method for cloning cDNA from mRNA encoding human-originated PGIS comprises the following steps.

Cells producing (secreting) human-originated PGIS, such as human aorta endothelial cells are cultured and mRNA encoding said PGIS is prepared from the culture thereof. mRNA is prepared by, for example, applying entire RNA prepared by a known method such as guanidine thiocyanate method [Chirgwin, J.M. et al., Biochem., *18*, 5294 (1979)], heat phenol method and AGPC to affinity chromatography using oligo(dT)-cellulose or poly U-sepharose.

Using the obtained mRNA as a template, cDNA chain is synthesized by a known method using a reverse transcriptase [e.g., the method of Okayama, H. et al: Okayama, H. et al., Mol. Cell. Biol., *2*, 161 (1982) and ibid. *3*, 280 (1983)], and the method of Gubler, U. and Hoffman, B.J.: Gubler, H. and Hoffman, B.J., Gene, *25*, 263 (1983)], thereby converting the same to a double stranded cDNA. This cDNA is inserted into a plasmid vector or a phage vector, with which *Escherichia coli* is transformed, or transfected after *in vitro* packaging, to prepare cDNA library.

The plasmid vector used here is not subject to any particular limitation as long as it can be retained by replication in the host, and the phage vector is not limited either as long as it can proliferate in the host. Examples of the conventionally-used cloning vector include pUC119, λgt10 and λgt11. When immunological screening to be mentioned later is to be employed, the vector preferably contains a promoter capable of expressing the PGIS gene in the host.

The method for insertion of a cDNA into plasmid is exemplified by a method described in Maniatis, T. et al [Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, p. 239 (1982)]. The method for insertion of a cDNA into phage vector includes the method of Hyunh, T.V. et al [DNA Cloning, a practical approach, *1*, 49 (1985)]. For simplification, a commercially available ligation kit (e.g., those manufactured by Takara Shuzo) can be used. The

recombinant plasmid and phage vector thus obtained are introduced into a suitable host such as prokaryotic cells (e.g., *E. coli* HB101, DH5 and MC1061/P3).

The method for introducing a plasmid into a host includes calcium chloride method and calcium chloride/rubidium chloride method described in Maniatis, T. et al [Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, p. 239 (1982)] and electroporation method. The method for introducing a phage vector into a host is exemplified by a method comprising *in vitro* packaging of phage DNA and introducing same into proliferated host cells. *In vitro* packaging can be carried out easily by using a commercially available *in vitro* packaging kit (e.g., product of Stratagene and product of Amersham).

The cDNA encoding the PGIS of the present invention can be isolated from the cDNA library prepared by the above method, by a combination of general cDNA screening methods.

Such methods include, for example, a method wherein an oligonucleotide considered to be corresponding to the amino acid sequence of human PGIS is chemically synthesized separately and labeled with ^{32}P to give a probe, and a clone having the desired cDNA is screened by a known colony hybridization [Crunstein, M. and Hogness, D.S., Proc. Natl. Acid. Sci. USA, 72, 3961 (1975)] or plaque hybridization [Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, p. 239 (1982)]; and a method wherein PCR primer is prepared and a specific region of PGIS is amplified by PCR method, which is followed by selecting a clone having a DNA fragment encoding said region. When a cDNA library prepared using a vector (e.g. λgt11 phage vector) capable of expressing cDNA is used, the objective clone can be selected based on an antigen-antibody reaction using the PGIS antibody of the present invention to be mentioned later. When large amounts of clone are treated, screening based on PCR is preferable.

The nucleotide sequence of DNA thus obtained can be determined by Maxam-Gilbert method [Maxam, A.M. and Gilbert, W., Proc. Natl. Acad.

Sci. USA., 74, 560 (1977)] or synthetic dideoxynucleotide chain termination method using phage M13 [Sanger, F. et al, Proc. Natl. Acad. Sci. USA., 74, 5463-5467 (1977)]. The PGIS gene can be obtained by cleaving all or part thereof from the clone obtained above by using a restriction enzyme and the like.

(2) A preparation method comprising isolating DNA encoding PGIS from genomic DNA of human aorta vascular cells includes, for example, the following method.

Human aorta vascular cells are lysed preferably using SDS or protenase K, and DNA is deproteinized by repetitive extraction with phenol. RNA is preferably digested with ribonuclease. The obtained DNA is partially digested with a suitable restriction enzyme and the obtained DNA fragment is amplified by a suitable phage or cosmid to form a library. Then, the clone having the desired sequence is detected by, for example, a method using a DNA probe with a radioactive label, and a whole or partial PGIS gene is cleaved from said clone by using a restriction enzyme and the like.

(3) The DNA of the present invention can be prepared by chemical synthesis by a conventional method based on the nucleotide sequence depicted in Sequence Listing Sequence No. 11.

The present invention further relates to a recombinant vector comprising DNA encoding the above-mentioned PGIS. The recombinant vector of the present invention is not particularly limited as long as it can be retained by replication or self-proliferation in various prokaryotic and/or eukaryotic host cells, and includes plasmid vector and phage vector.

The recombinant vector can be easily prepared by ligating the DNA encoding human-originated PGIS of the present invention with a commercially available recombinant vector (plasmid DNA and bacteriophage DNA) by a conventional method. Usable recombinant vector includes, for example, *Escherichia coli*-originated plasmids pBR322, pBR325, pUC12 and pUC13; yeast-originated plasmids pSH19 and pSH15; and *Bacillus subtilis*-originated plasmids pUB110, pTP5 and

pC194. Examples of phage include bacteriophage such as λ phage, and animal or insect viruses such as retrovirus, vaccinia virus, nuclear polyhedrosis virus and adenovirus [e.g. pVL1392, pBK283, *Autographa californica* nuclear polyhedrosis virus (AcNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV)].

When production of PGIS by the expression of the PGIS gene is aimed, an expression vector is useful. The expression vector is not particularly limited as long as it expresses the PGIS gene in various prokaryotic and/or eukaryotic host cells and is capable of producing proteins. Preferred are that derived from insect virus which infects insect cells and produces PGIS in said cells, and that derived from animal virus which infects animal cells and produces PGIS in said cells.

When bacteria, particularly *Escherichia coli*, is used as the host cell, the expression vector generally consists of at least promoter-operator region, initiation codon, DNA encoding the PGIS of the present invention, termination codon, terminator region and replicon.

When yeast, animal cell or insect cell is used as the host cell, the expression vector preferably consists of at least promoter, initiation codon, DNA encoding the polypeptide of the present invention and termination codon. It may contain DNA encoding signal peptide, enhancer sequence, non-translation region on the 5' or 3' side of the polypeptide of the present invention, splicing junction, polyadenylation site, selection marker region, replicon and the like.

The promoter-operator region for expressing the polypeptide of the present invention in bacteria contains promoter, operator and Shine-Dalgarno (SD) sequence such as AAGG. When the host is *Escherichia coli*, the region preferably contains, for example, Trp promoter, lac promoter, recA promoter, λPL promoter and lpp promoter. The promoter for expressing PGIS in yeast includes, for example, PHO5 promoter, PGK promoter, GAP promoter and ADH promoter, and when the host is bacteria belonging to the genus *Bacillus*, SL01

promoter, SPO2 promoter and penP promoter can be used. When the host is eukaryotic cells such as animal cells, examples of the promoter include, but not limited to, SV40-derived promoter, retrovirus promoter, heat shock promoter, polyhedron promoter that a nuclear polyhedrosis virus has, cytomegalovirus promoter, adenovirus promoter and ß-actin promoter. The use of an enhancer is also effective for the expression.

Preferable initiation codon includes, for example, methionine codon (ATG).

The termination codon is exemplified by conventional termination codons such as TAG and TGA.

As the terminator region, conventional intact or synthetic terminator can be used.

By replicon is meant a DNA capable of reproducing the entire DNA sequence in the host cell, and exemplified by naturally occurring plasmid, artificially modified plasmid (DNA fragment prepared from naturally occurring plasmid) and synthetic plasmid. Examples of preferable plasmid include plasmid pBR322 and artificial modification thereof (DNA fragment obtained by treating pBR322 with a suitable restriction enzyme) in the case of *E. coli*; yeast 2 μ plasmid and yeast chromosomal DNA in the case of yeast; and plasmid pRSVneo ATCC 37198, plasmid pSV2dhfr ATCC 37145, plasmid pdBPV-MMTneo ATCC 37224 and plasmid pSV2neo ATCC 37149 in the case of mammalian cell.

Enhancer sequence, polyadenylation site and splicing junction site can be those conventionally used by artisan, such as respective ones derived from SV40.

As the selection marker, conventional ones can be used according to a conventional method. Examples thereof include a gene resistant to antibiotic such as tetracycline, ampicillin and kanamycin.

The expression vector of the present invention can be prepared by ligating at least the above-mentioned promoter, initiation codon, DNA encoding PGIS of the present invention, termination codon and terminator region sequentially and cyclically into a suitable

replicatable unit. For this end, suitable DNA fragments such as linker and other restriction sites can be used by a conventional method such as digestion with restriction enzyme and ligation using T4DNA ligase on demand.

The transformant of the present invention can be prepared by introducing the above-mentioned expression vector into a host cell.

Examples of the host cell include microorganisms such as bacteria (e.g. bacteria belonging to the genera *Escherichia* and *Bacillus*), yeast such as those belonging to the genus *Saccharomyces*, animal cells and insect cells. Specifically exemplified are *Escherichia coli* K12DH1, M103, JA221, HB101, C600, XL-1 Blue and JM109 as the bacteria belonging to the genus *Escherichia*; and *Bacillus subtilis* 207-21 as the bacteria belonging to the genus *Bacillus*. Examples of the yeast include *Saccharomyces cerevisiae* AH22, AH22R$^-$, NA87-11A and DKD-5D. Examples of animal cell include simian cell COS-7, Vero, Chinese hamster cell CHO, mouse L cell, human FL cell and human 293 cell. Examples of insect cell include BmN4 and Sf9. Preferred are insect cells and animal cells.

The preferred host cell for cloning the DNA sequence and constructing the vector is generally a prokaryotic cell. The expression vector constructed is used to transform a suitable host cell. The host cell may be a prokaryotic cell or an eukaryotic cell as well. Preferred are insect cells (e.g., BmN4 and Sf) and animal cells.

The expression vector is introduced (i.e., transformation which is used in a concept inclusive of transfection in the present invention) into host cells by a conventionally known method.

For example, in the case of bacteria (e.g. *Escherichia coli* and *Bacillus subtilis*), the method of Cohen et al [Proc. Natl. Acad. Sci. USA., *69*, 2110 (1972)], protoplast method [Mol. Gen. Genet., *168*, 111 (1979)] or competent method [J. Mol. Biol., *56*, 209 (1971)] may be used; in the case of *Saccharomyces cerevisiae*, the method of Hinnen et al [Proc. Natl. Acad. Sci. USA., *75*, 1927 (1978)] or lithium

method [J. Bacteriol., *153*, 163 (1983)] may be used; in the case of animal cells, the method of Graham [Virology., *52*, 456 (1973)], lipofectin method or HVJ-liposome method [Hypertension, *21*, 894-899 (1993)] may be used; and in the case of insect cells, the method of Summers et al [Mol. Cell. Biol., *3*, 2156-2165 (1983)] may be used for transformation.

The human-originated PGIS of the present invention can be prepared by culturing, in a nutrient medium, a transformant (which term is used in a concept inclusive of transfectant in the present invention) comprising the expression vector prepared as in the above.

The nutrient medium preferably contains carbon source, inorganic nitrogen source or organic nitrogen source necessary for the growth of host cell (transformant). Examples of carbon source include glucose, dextran, soluble starch and sucrose; examples of inorganic nitrogen source or organic nitrogen source include ammonium salts, nitric acid salts, amino acid, corn steep liquor, peptone, casein, meat extract, soybean meal and potato liquid extract. When desired, other nutrients such as inorganic salt (e.g. calcium chloride, sodium dihydrogenphosphate and magnesium chloride), vitamins, and antibiotics such as ampicillin and kanamycin may be added to the medium.

Culture is carried out according to the method known in the pertinent field. Culture conditions such as temperature, pH of the medium and culture time are appropriately determined so that the maximum potency of PGIS can be obtained.

Specific media and culture conditions to be employed according to the host cell are exemplified in the following, which are not limitative.

When the host is bacteria, Actinomyces, yeast or filamentous fungus, for example, liquid media containing the above-mentioned nutrient sources are appropriate. Preferred is a medium having a pH of 5-8.

When the host is *Escherichia coli*, preferable medium is M9 medium

[Miller, J., Exp. Mol. Genet., p. 431, Cold Spring Harbor Laboratory, New York (1972)]. In this case, culture is performed with aeration and agitation as necessary, at 14-43°C for about 3 to 24 hours.

When the host is bacteria belonging to the genus *Bacillus*, culture is performed with aeration and agitation as necessary, at 30-40°C for about 16 to 96 hours.

When the host is yeast, the medium is exemplified by Burkholder minimum medium [Bostian, K. L. et al, Proc. Natl. Acad. Sci. USA, 77, 4505 (1980)] which preferably has a pH of 5-8. Culture is generally performed at about 20-35°C for about 14 to 144 hours with aeration and agitation where necessary.

When the host is animal cell, the medium is exemplified by MEM medium containing fetal calf serum at about 5-20% [Science, *122*, 501 (1952)], DMEM medium [Virology, *8*, 396 (1959)], RPMI1640 medium [J. Am. Med. Assoc., *199*, 519 (1967)] and 199 medium [Proc. Soc. Exp. Biol. Med., *73*, 1 (1950)]. The pH of the medium is preferably about 6-8, and culture is generally performed at about 30-40°C for about 15-60 hours with aeration and agitation where necessary.

When the host is insect cell, the medium is exemplified by Grace's medium containing fetal calf serum [Proc. Natl. Acad. Sci. USA, *82*, 8404 (1985)] which preferably has a pH of about 5-8. Culture is generally performed at about 20-40°C for about 15 to 100 hours with aeration and agitation where necessary.

The human-originated PGIS of the present invention can be recovered as in the following from the culture obtained above.

That is, when the human-originated PGIS is present in the liquid portion of the culture, the culture thus obtained is subjected to filtration or centrifugation to separate culture filtrate (supernatant), and PGIS is purified and separated from said culture filtrate by a conventional method employed for purifying and isolating natural or synthetic proteins.

The method for purification and isolation includes, for example, a method utilizing the solubility, such as salting out and solvent

precipitation, a method utilizing the difference in molecular weights such as dialysis, ultrafiltration, gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a method utilizing charge such as ion exchange chromatography and hydroxyapatite chromatography, a method utilizing specific affinity such as affinity chromatography, a method utilizing the difference in hydrophobicity such as reversed phase high performance liquid chromatography and a method utilizing difference in isoelectric point such as isoelectric focusing.

When the human-originated PGIS is present in the periplasm or cytoplasm of the cultured transformant, the culture is subjected to a conventional method such as filtration and centrifugation to collect the cells; the cells are suspended in a suitable buffer and subjected to lysis of cell wall and/or cell membrane by ultrasonication, using lysozyme or by freeze-thawing; and the membrane fraction containing PGIS is obtained by centrifugation or filtration. Said membrane fraction is solubilized with surfactant such as Triton to give a crude solution. The crude solution is treated by a conventional method as exemplified supra to isolate and purify PGIS of the present invention.

The present invention also relates to an antibody having a reactivity with the above-said human-originated PGIS. The antibody of the present invention encompasses both the polyclonal antibody and monoclonal antibody having the above-mentioned properties. The antibody of the present invention can be obtained by a conventional method.

For example, the monoclonal antibody of the present invention can be prepared from hybridoma produced by so-called cell fusion. That is, fused hybridoma is formed from the antibody-producing cell and bone marrow cell; said hybridoma is cloned; and a clone is selected which produces an antibody having a specific affinity for an antigen, i.e. a polypeptide having part or whole of the human-originated PGIS amino acid sequence. The procedure therefor may be known methods except the use of the human-originated PGIS of the present invention as an immunizing antigen.

The immunogen can be used for immunizing animals after admixing with, for example, complete Freund adjuvant. Examples of the animal include mouse, rat and rabbit. The animals are immunized by subcutaneous, intramuscular or intraperitoneal injection of about 5-200 μg/injection. The immunization includes 1-4 times of immunization at about every 1-2 weeks from the initial immunization and final immunization at about 1-4 weeks thereafter. When about 3-5 days have passed since final immunization, antibody-producing cells are separated from the immunized animal. The antibody-producing cells are exemplified by spleen cells and lymph node cells.

The bone marrow cells are, for example, those derived from mouse, rat and human. Examples thereof include mouse myeloma P3 · X63 · Ag8, P3 · X63 · Ag8-U1, P3 · NS1-Ag4, SP2/0-Ag14 and X63-Ag8 · 653. It is preferable that the antibody-producing cells and bone marrow cells be derived from the same species of animals.

Cell fusion is performed by the method described in, for example, Nature, vol. 266, p. 550 (1977) or an analogous method. Specifically, it is performed using 30-50% polyethylene glycol having an average molecular weight of 1,000-4,000 at 30-40°C for about 1-3 minutes.

The cells obtained by cell fusion are subjected to screening for a clone which produces the desired monoclonal antibody. That is, the cells are cultured in, for example, a microplate and the antibody titer of the culture supernatant in the well in which cell growth was acknowledged is determined by, for example, enzyme antibody method to obtain the well in which suitable antibody has been produced. Cloning by, for example, limiting dilution from such well gives clones. The monoclonal antibody of the present invention can be obtained by culturing said hybridoma cell clone by conventional culture method, high density culture method or spinner-flask culture method and purification thereof by affinity chromatography using protein A-bound carrier or anti-mouse immunoglobulin-bound carrier.

Alternatively, the cultured hybridoma cells are intraperitoneally

injected to the mouse of the same species which has been previously treated with pristance, and ascites obtained is subjected to salting out with ammonium sulfate and DEAE ion exchange chromatography to give purified IgG fraction containing the same.

The DNA encoding the human-originated PGIS of the present invention can be used for gene therapy.

The DNA encoding the human-originated PGIS of the present invention or a recombinant vector comprising said DNA is introduced into human or other animals, whereby PGIS is produced in the human or other animals to promote production of PGI_2. The promoted PGI_2 production in turn enables treatment (therapeutic treatment or improvement of symptoms) of the diseases induced by a low production of PGI_2. Examples of the diseases induced by the low production of PGI_2 include cardiovascular diseases such as thrombosis, myocardial infarction, arteriosclerosis and angina pectoris. The recombinant vector may be introduced into human or other animals in the form of cells transformed with said recombinant vector.

The gene therapy utilizing the gene (inclusive of DNA and recombinant vector) of the present invention permits setting an appropriate environment in which the gene of the present invention introduced into a human or other animal can fully show its function. The treatment can be given by a conventional method as long as it intends expression of desired effects of the gene of the present invention. Such method is exemplified by virological means utilizing retrovirus vector or adenovirus vector, physical means for introducing gene by particle gun method or by using naked DNA, and chemical means such as lipid method [Molecular Medicine, vol. 30, No. 12, p. 1526 (1993); *Jikken Igaku*, vol. 12, No. 3, p. 15, 28 and 40 (1994); Proc. Natl. Acad. Sci. USA, *92*, 1137 (1995)]. A method using an adenovirus vector which can be used for the gene therapy of cystic fibrosis and which is known to permit efficient introduction of gene into differentiated cells and tissues and expression therein, and a method using a fusogenic liposome which allows introduction of

optional gene into tissue cells *in vivo* are preferable for the gene treatment of the present invention.

The dose of the DNA or recombinant vector of the present invention is subject to appropriate change according to sex, age and body weight of patients, the kind of disease and symptoms thereof, and administration route. For example, 100 μg-10 mg of DNA is generally administered.

The DNA and recombinant vector of the present invention are administered by intravenous injection, transmucosal administration, oral administration using enteric-coated agents, or topical administration, with preference given to topical administration using catheter and the like.

The DNA encoding human-originated PGIS and recombinant vector comprising said DNA of the present invention are admixed with conventional, pharmaceutically acceptable carrier, excipient, diluent, extender, disintegrator, stabilizer, preservative, buffer, emulsifier, flavor, coloring, sweetener, thickener, elixir, solubilizer and other additives such as water, salt solution, phosphate buffer, vegetable oil, ethanol, polyethylene glycol, glycerol, gelatin, lactose, glucose, mannitol, starch, sucrose, magnesium stearate, hydroxypropylcellulose, talc, lanolin and petrolatum, and can be use used in the form of injection, tablet, powder, capsule, enteric-coated agent, ointment, suspension, emulsion, spray, inhalant, collunarium and the like.

A pharmaceutical composition comprising the DNA or recombinant vector comprising said DNA of the present invention can be administered to mammals such as human, mouse, rat, rabbit, pig, cow, sheep, dog and cat.

Effects of the Invention

The present invention gives the first clarification of the amino acid sequence of human-originated PGIS and nucleotide sequence of DNA encoding the enzyme having said sequence. Based on the elucidation of such amino acid sequence and nucleotide sequence, the present

invention provides a method for preparing PGIS by genetic engineering and an expression system related thereto.

The PGIS and DNA encoding same of the present invention are useful as reagents for

(1) the analysis of physicochemical property and biological property of PGIS at the molecular or genetic level,

(2) the analysis of the mechanism of regulating PGIS production and the mechanism of regulating PGI_2 production by PGIS, and

(3) the investigation of the cause of various cardiovascular diseases considered to be induced by the production imbalance between PGI_2 and TXA_2, and analysis at the molecular or genetic level for the development of therapeutic agent for said diseases.

In addition, they are useful as diagnostics for determining the *in vivo* tissue expression level and distribution of PGIS or mRNA thereof.

Moreover, they can be used as therapeutic agents for various cardiovascular diseases such as thrombosis, myocardial infarction, arteriosclerosis and angina pectoris, which increase the production level of PGI_2 based on lesion-specific introduction, into human and other animals, of PGIS, DNA encoding PGIS, fragment thereof or modified product thereof.

The expression system of PGIS comprising a recombinant vector containing DNA encoding the human-originated PGIS of the present invention, and a host cell transformed with said vector is useful for the production by genetic engineering, which enables easy and efficient mass production of human-originated PGIS.

In addition, the human-originated PGIS antibody of the present invention serves well for the purification of human-originated PGIS and the immunohistochemical analysis of the cause of a disease (specific staining of various tissues such as uterus, heart, skeletal muscle, lung and prostate).

The plasmid, enzyme such as restriction enzyme, T4DNA ligase and other substances to be used in Examples of the present invention are

commercially available and can be used according to a conventional method. The procedures for cloning of cDNA, determination of nucleotide sequence, transfection of host cell, culture of transfectant, harvesting and purification of PGIS from obtained culture, and obtainment of antibody are well known to those skilled in the art, or can be known from literatures.

The pHPGIS36 (PBJT-BA-4, deposit number FERM BP-4653) and pHPGIS135 (PBJT-BA 5, deposit number FERM BP-4654) used in the present invention are at international deposit at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology.

Examples and Reference Examples

The present invention is described detailedly in the following by way of Examples and Reference Examples, to which the present invention is not limited.

Example 1: Determination of cDNA nucleotide sequence

(1) Preparation of λhPGIS141

Human genomic library (Genomic lung fibroblast cell line, W I 38, manufactured by Clone-Tech) was seeded at about 2×10^5 PFU, and screened by plaque hybridization using, as a probe, a bovine cDNA prepared in advance by the inventor (see Tanabe, T., Hara, S., Miyata, A., Brugger, R., and Ullrich, V. (1993) in Abstract book of 3rd international conference on eicosanoid and other bioactive lipids in cancer, inflammation and radiation Injury, pp. 137).

As a result, four positive signals were obtained, one of which was isolated to a single plaque. Liquid culture thereof resulted in mass preparation of phage DNA. After purification, it was digested with various restriction enzymes, followed by mapping. A fragment comprising exon was identified by Southern hybridization, which was followed by structural analysis by DNA sequencing to confirm that the finally-isolated clone (λhPGIS141) coded for human PGIS.

The λhPGIS141 thus obtained was structurally analyzed by restriction enzyme site mapping and nucleotide sequence determination,

and it was found that λhPGIS141 contained the region corresponding to a 673rd - 855th nucleotide sequence of bovine PGIS cDNA (SQ No. 8).

Based on the nucleotide sequence of λhPGIS141 cDNA fragment thus obtained, primers [SQ No. 1 : P1 primer (674-689), SQ No. 2 : P2 primer (699-718), SQ No. 3 : P3 primer (696-713), SQ No. 4 : P4 primer (805-822)] having the sequences depicted in Sequence List SQ Nos. 1-4 were synthesized.

(2) Amplification of cDNA by PCR method

The 3'-downstream region and 5'-upstream region of cDNA were amplified by PCR method (Biochem. Biophys. Res. Commun. *178*, 1479-1484 (1991)) using said primers and poly(A)$^+$ RNA (mRNA) from 1 μg of human aorta vascular endothelial cells (hereinafter referred to as HAEC, manufactured by Kurabo) as a template.

For amplification of cDNA corresponding to the 3'-downstream region, cDNA was primed with a dT_{17} adapter (5'-GACTCGAGTCGACATCGA-$(T)_{17}$-3', SQ No. 5), and elongated to give a first cDNA chain which was amplified with P1 primer (674-689) and the adapter primer (SQ No. 6), and then with P2 primer (699-718) and the adapter primer (SQ No. 6). The 5'-upstream region of the cDNA was amplified using a 5' RACE system (GIBCO BRL). According to the protocol, homomeric dC tail was added to the first cDNA chain and a second cDNA chain was formed using an adapter primer (5'-$(CUA)_4$ GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') (SQ No. 7). The fist step amplification was performed using P4 primer and the adapter primer (SQ No. 7). The second step amplification was performed using P3 primer and the adapter primer (SQ No. 7). The PCR method was repeated 35 cycles according to the following cycloprofile.

Denaturation	94°C,	1 minute
Annealing	54°C,	1 minute
Elongation	72°C,	3 minutes

The respective PCR products (3'-downstream region amplification product and 5'-downstream region amplification product) were partially taken out and purified by electrophoresis using 1% agarose

gel. Southern hybridization was applied using bovine cDNA (pBPGISI) as a probe, and DNA was extracted from the band which cross-hybridized to said probe. The obtained DNA was cloned into pBluescriptII SK(-).

That is, cloning and screening were performed by the following steps:

(1) cleaving out the band which showed a signal from a gel, after electrophoresis
(2) agarase digestion at 40°C for one hour (agarase 1 unit/100 μl gel)
(3) extraction of DNA with phenol and subsequent ethanol precipitation
(4) dissolving said DNA ethanol precipitate in sterile water and treating with polynucleotide kinase at 37°C for one hour
(5) end repairing with Klenow fragment (16°C, 1 hr)
(6) ligation using Takara ligation kit
(7) transformation by a conventional method
(8) sewing in a plate
(9) forming a replica by a conventional method and
(10) colony hybridization of nitrocellulose filter of the replica by a conventional method, using bovine PGIS cDNA as a probe

The hybridization was performed at 60°C in 6×SSC [1×SSC containing 0.15 M NaCl, 15 mM sodium citrate (pH 7.0)], 5×Denhardt's solution, 250 μg/ml salmon sperm DNA, 0.1% SDS and cDNA fragment (10^6 cpm/ml) labeled by random priming method. The filter obtained was washed twice with 3×SSC and 0.1% SDS at room temperature for 5 minutes and twice with 0.1×SSC and 0.1% SDS at 50°C for 15 minutes. The filter was air-dried, and exposed to Fuji X ray film using a intensifying screen at -80°C for 12-16 hr.

The obtained DNA insert was subcloned into pBluescriptII SK(-). By these steps, a clone (pHPGIS135) containing 3'-downstream region DNA of human-originated PGIS and a clone (pHPGIS36) containing 5'-upstream region DNA of human-originated PGIS were obtained. Then, the nucleotide sequence of the DNA insert of respective clones was determined by the Sanger method [Sanger, F., Nickle,, S., and Coulson,

A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467] using Taq dye primer cycle sequence kit (manufactured by Applied Biosystems) and Model 373A DNA sequencer (manufactured by Applied Biosystems). As a result, it was found that pHPGIS36 clone had, as a DNA insert sequence, a 740 bp nucleotide sequence (SQ No. 9) of cDNA of human PGIS, having an adapter sequence on the 5' side, based on which partial amino acid sequence of PGIS comprising 238 amino acid residues wherein ATG is the translation initiation sequence (Met) was identified.

It was also found that pHPGIS135 clone comprised, as a DNA insert sequence, a 1277 bp nucleotide sequence (SQ No. 10) of cDNA of human PGIS, having an adapter sequence on the 3' side, based on which partial amino acid sequence of PGIS on the carboxyl side region starting from 226th aspartic acid was identified. The nucleotide sequence of human PGIS cDNA contained in pHPGIS36 clone and the amino acid sequence deduced therefrom are depicted in SQ No. 9 in the Sequence Listing to be mentioned later, and the nucleotide sequence of human PGIS cDNA contained in pHPGIS135 clone and the amino acid sequence deduced therefrom are depicted in SQ No. 10 therein. Fig. 1 shows a restriction enzyme map of human PGIS cDNA and the region of human PGIS cDNA, which corresponds to the DNA contained in λ hPGIS141, pHPGIS36 and pHPGIS135. Fig. 2 shows a restriction enzyme map of pHPGIS36 and Fig. 3 shows a restriction enzyme map of pHPGIS135.

Human PGIS cDNA obtained by the above-mentioned cloning had a consensus sequence of the initiation codon of eukaryotic shown by Kozak et al [Nucleic Acids Res. 12, 857-872 (1984)] at around the translation initiation codon, and TGA codon corresponding to the termination codon at 500 codons therefrom. Based on these facts, it was found that the cDNA of the cloned human PGIS comprised 1977 bp comprising 1500 bp encoding 500 amino acid residues, as shown in SQ No. 12, and the molecular weight of the protein coded thereby was speculated to be about 57,000.

Comparison of the amino acid sequence encoded by said DNA with the amino acid sequence of bovine-originated PGIS separately cloned by the present inventor revealed an about 88% homology. The study of bovine PGIS by the present inventor found that the bovine PGIS had a 31% homology with cholesterol 7α-hydroxylase belonging to the cytochrome P450 7 family (CYP7), and the region around the 441st Cys residue, which is heme-binding site (fifth ligand) of cytochrome P450, was reserved. The human PGIS similarly reserved the amino acid sequence corresponding to said region, and this region is considered to play an important role in the PGIS activity.

Although the bovine PGIS had a 31% homology with cholesterol 7α-hydroxylase, it had only a 16% homology with human thromboxane synthase belonging to the cytochrome P450 family and a not more than 40% homology with any of the known cytochrome P450 proteins. It is postulated, therefore, that it is a new family in the cytochrome P450 super family, and human PGIS also belongs to this new family.

A search for such structural correlation in activity is indispensable for the study and development of pharmaceutical products. Such search is accomplished only after the primary structure of human PGIS has been clarified. Accordingly, the present invention which discloses the primary structure of human PGIS for the first time is extremely important and significant for the research, and from industrial aspect as well.

Example 2: Expression of human PGIS

(1) Construction of expression vector for human PGIS

A cDNA insert region is cleaved out respectively from the obtained pHPGIS36 clone and pHPGIS135 clone using a suitable restriction enzyme, and purified. The both fragments obtained were thermally denatured (95°C for 10 minutes), followed by annealing. cDNA is replicated using a DNA polymerase to the both directions toward 5' and 3' from the overlapped region as the synthesis initiation region. Using the obtained whole length cDNA as a template, a primer is synthesized from each region of initiation codon

or termination codon and PCR is performed. On this occasion, a suitable restriction enzyme site is constructed as an anchor site at 3' of the primer.

The PCR product thus obtained is purified, the nucleotide sequence of which is confirmed, and the product is digested with BamHI and SmaI (BglII) to give a BamHI-SmaI (BglII) fragment. Said BamHI-SmaI (BglII) fragment is introduced into the BamHI-SmaI site of pVL1393 expression vector previously treated with BamHI-SmaI. The recombinant plasmid thus formed (PGIS7) is characterized by restriction enzyme mapping and DNA sequence analysis.

(2) Baculovirus expression system

Sf9 cells (manufactured by In Vitrogen) are mono-layer cultured in a Grace's insect medium containing 10% fetal calf serum, 0.33% yeastolate and 0.33% lactoalbumin hydrolysate at 27℃. For the production of a recombinant virus, Sf9 cell (1.5×10^6 cells) recombinant plasmid (PGIS7, 50 μg) and wild type baculovirus DNA (AcNPV; 1 μg) are mixed and transfected by calcium phosphate precipitation method. The recombinant baculovirus is isolated and amplified by a combination of plaque assay and slot hybridization using a ^{32}P-labeled cDNA fragment of PGIS as a probe.

Said Sf9 cells are infected with wild type baculovirus or recombinant baculovirus. At 3 days after the infection, cells are collected (2×10^8 cells) and incubated for 5 hours in a serum-containing medium with or without 10 μM hemin.

The obtained cells are washed with phosphate-buffered saline and preserved at -80℃. The microsomal fraction of the cell is prepared according to the method of Haurand and Ullrich et al. (J. Biol. Chem. 260, 15059-15067). The obtained cells (2×10^8 cells) are homogenized in a solution (20 ml) of 10 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 5 mM glucose, 0.1 mM dithiothreitol (DTT), 1.15% KCl, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor and 44 μg/ml phenylmethylsulfonyl fluoride, and subjected to ultrasonication (30 seconds, 4 times) using a Branson

sonifier model 450.

The obtained homogenate is centrifuged at 7,000×g for 15 minutes, and the obtained supernatant is centrifuged at 105,000×g for 60 minutes. The sediment obtained is suspended in 10 mM potassium phosphate buffer (3 ml, pH 7.0) containing 20% glycerol, 1 mM DTT and 1 mM EDTA by sonication. The protein concentration is determined by Lowry method using bovine serum albumin as a standard, and a solution for immunoblot analysis and PGIS assay at 5 mg/ml is prepared.

(3) Western immunoblot analysis

The infected Sf9 cells and human platelet microsomal fraction are subjected to 10% SDS-PAGE according to the method of Laemmli [Nature 227, 680-685 (1979)]. The migrated protein is electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) according to the method of Towbin et al. [Proc. Natl. Acad. Sci. USA 76,4350-4354 (1979)]. Tris-HCl buffered saline (TBS) (pH 7.4) containing 10% equine serum is pretreated at room temperature for 30 minutes, and the blot membrane is incubated with polyclonal antibody against bovine PGIS in TBS containing 3% skim milk.

After washing with TBS containing 0.05% Tween 20, the membrane is incubated in TBS containing 3% skim milk at 37°C for 30 minutes together with anti-mouse IgG equine antibody conjugated with horseradish peroxidase (manufactured by Vector Laboratories). After thorough washing with TBS containing 0.05% Tween 20, the band showing positive immunological response is detected using an immunostaining HRP kit (manufactured by Konica).

Example 3: Expression of human PGIS in cultured animal cell

(1) Preparation of whole length human PGIS cDNA

The obtained pHPGIS36 clone was cleaved out with restriction enzymes SalI and NspI and purified to give a SaLI-NspI fragment. The pHPGIS135 clone was cleaved out with restriction enzymes PstI and BamHI and purified to give a PstI-BamHI fragment. Furthermore, primers [SQ No. 13 : P5 primer (676-699), SQ No. 14 : P6 primer (832-

855)] having sequences depicted in Sequence Listing Sequence Nos. 13 and 14 were synthesized based on the nucleotide sequence of λhPGIS141. Using these primers and λhPGIS141 as a template, a middle stream region of human PGIS cDNA was amplified by PCR method, cleaved with restriction enzymes NspI and PstI, purified and confirmed for the nucleotide sequence and used as an NspI-PstI fragment. These SalI-NspI fragment, PstI-BamHI fragment and NspI-PstI fragment were bound and introduced into the SalI-BamHI site of pBluescriptII SK+ (manufactured by STRATAGENE) previously treated with SalI-BamHI, whereby a plasmid (pHPGIS1) containing the whole length human PGIS cDNA was prepared. Fig. 4 shows the restriction enzyme map of pHPGIS 1.

(2) Construction of human PGIS expression vector for cultured animal cell

Human PGIS cDNA insert region was cleaved out from the obtained pHPGIS1 clone with restriction enzymes SalI and BamHI and purified to give a SalI-BamHI fragment. This SalI-BamHI fragment was introduced into the SalI-BamHI site of pCMV7 expression vector [supplied by Dr. David W. Russel, University of Texas Southwestern Medical Center, Cell, 75, 187-197 (1993); J. Biol. Chem., 264, 8222-8229 (1989)] previously treated with SalI-BamHI, whereby a human PGIS expression vector (pCMV-HPGIS 1) for cultured animal cell was prepared. Fig. 5 shows the restriction enzyme map of pCMV-HPGIS 1.

(3) Expression of human PGIS in cultured animal cell

Human fetus kidney-derived 293 cells (manufactured by Dainippon Pharmaceutical Co., Ltd.) were sewn in a 60 mm dish at 3×10^5 cells, and mono-layer cultured at 37°C for 24 hours in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Then, a recombinant plasmid (pCMV-HPGIS 1, 3 μg) and pVA1 [adenovirus VA1 gene, 3 μg : supplied by Dr. David W. Russel, University of Texas Southwestern Medical Center; Mol. Cell. Biol., 7, 549-551 (1987)] were mixed and transfected by lipofectin method (GIBCO BRL). At 40 hours after the

transfection, the cells were washed with phosphate-buffered saline and collected. The cells were suspended in 10 mM calcium phosphate buffer (pH 7.0) containing 10 mM EDTA, 10 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM glucose, 0.1 mM dithiothreitol (DTT), 1.15% KCl, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 10 μg/ml soybean trypsin inhibitor, and subjected to ultrasonication (10 seconds, 10 times) using ASTRASON™ Model XL2020.

The obtained homogenate was centrifuged at 100,000×g for 60 minutes and the obtained sediment was suspended in 10 mM calcium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM PMSF, 20% glycerol and 0.1 mM DTT. The protein concentration of the obtained sample was determined using a BCA (bicinchoninic acid) protein concentration determination kit (manufactured by PIERCE) using bovine serum albumin as a standard. The PGIS activity of the obtained sample was determined by reacting same with ^{14}C-labeled PGH_2 (5 nmole) as a substrate at 24°C for 2 minutes, separating 6-keto-$PGF_1\alpha$, which is a metabolite of the produced PGI_2, by thin layer chromatography, and detecting the radioactivity of the 6-keto-$PGF_1\alpha$. Fig. 7 shows the detected PGIS activity, Fig. 8 shows PGIS activity of positive control (bovine platelet microsomes) and Fig. 9 shows the analysis results, by thin layer chromatography, of negative control wherein pCMV7 alone was introduced.

As the result of the determination using a sample prepared from the cell into which an expression vector incorporating human PGIS cDNA had been introduced, a spot of 6-keto-$PGF_1\alpha$, which is a metabolite of PGI_2, was detected as shown by an arrow in Fig. 7. The results were the same as those obtained using bovine platelet microsome containing PGIS as a positive control (Fig. 8). In contrast, the determination using a sample prepared from the cell into which an expression vector without human PGIS cDNA had been introduced failed to detect a spot of 6-keto-$PGF_1\alpha$. The spot of PGH_2 was thicker (Fig. 9) than in Fig. 7 and Fig. 8. The above results mean that PGIS cDNA incorporated in the expression vector was

expressed as a recombinant protein (recombinant PGIS) having PGIS activity and this protein acted on PGH_2 to produce 6-keto-$PGF_{1\alpha}$ which is a metabolite of PGI_2.

Example 4: Expression of human PGIS in cultured animal cell

Human PGIS cDNA was bound to the XhoI site of the expression vector pUC-CAGGS [having an enhancer of cytomegalovirus and chiken ß-actin promoter] as shown in Fig. 6 [prepared according to the description in Gene 108, 193-200 (1991)] to construct an expression vector. Two kinds of vectors, i.e., this vector and a vacant vector without human PGIS cDNA, were introduced into vascular smooth muscle cells respectively prepared from rat aorta by HVJ-liposome method [Hypertension 21, 894-899 (1993)] and incubated in a serum-free medium [Dulbecco modified Eagle's medium (DMEM) containing 5×10^{-7} M insulin, 50 µg/ml transferin, 0.2 mM ascorbic acid, 100 U/ml penicillin and 100 µg/ml streptomycin] in a CO_2 incubator at 37°C for 2 days. Then, the medium was changed to a medium containing 1% or 5% fetal calf serum (FCS), and ^{3}H-thymidine was added 16 hours later. At 8 hours after the addition of thymidine, the thymidine uptake was determined by a conventional method [Cancer Immunol. Immunother. 24, 158-164 (1987)].

The results are shown in Fig. 10, wherein control was a cell into which a vacant vector was introduced and PGIS was a cell into which an expression vector bound with human PGIS cDNA was introduced.

Addition of serum to the vascular smooth muscle cell cultured in the absence of serum led to a promoted proliferation which increased thymidine uptake. In the vascular smooth muscle cell into which an expression vector ligated with human PGIS cDNA was introduced, thymidine intake, namely, proliferation, was significantly suppressed as compared to the cell into which a vacant vector was introduced. This result suggests the possibility of PGIS cDNA introduction suppressing abnormal growth of smooth muscle cells in vascular intima which is observed in arteriosclerosis and the like.

Example 5: Preparation of anti-PGIS polyclonal antibody

PGIS dissolved in 0.5 ml of phosphate-buffered saline (PBS) and an equivalent amount of adjuvant were emulsified and subcutaneously injected to rabbit. Thereafter, similar subcutaneous injection was given twice every 10 days, and blood was taken from the rabbit 10 days after the final subcutaneous injection. Anti-PGIS/IgG was purified and obtained from rabbit anti-PGIS serum prepared from the blood of said rabbit using protein A sepharose 4B (Bio-Rad).

Example 6: Preparation of anti-PGIS monoclonal antibody

① Mouse

Male inbred line BALB/c mice (5 weeks of age) were obtained and bred on standard pellet in an animal breeding chamber (23+1℃, 70% humidity) with optional watering.

② Immunogen

Human-originated purified PGIS was used. The human PGIS was prepared to a concentration of 1 mg/ml with Dulbecco PBS, dispensed into test tubes by 100 μg and freeze-preserved at -80℃ until use.

③ Immunizing method

Human PGIS 100 μg/0.5 ml and an equivalent amount of Freund's complete adjuvant were mixed. An emulsified antigen (20 μg) was administered to five male BALB/c mice (5 weeks of age) intraperitoneally and subcutaneously at dozen sites on the back every 2 weeks for 2 months. After the immunization for 2 months, antibody titer was measured, and the mice having high antibody titer were picked and applied with additional intraperitoneal administration of 50 μg, 100 μg or 200 μg thereof every other week.

After the immunization for 2 months, two different mice were intraperitoneally administered with 100 μg thereof after a blank of one month. One week later, 100 μg thereof was intravenously injected for additional immunization.

④ Cell fusion

At 3 days from the final immunization, the spleen of the BALB/c mice was removed to prepare suspensions of spleen cells in EMEM culture medium. The spleen cells were washed 4 times with EMEM

culture medium and counted.

For cell fusion, 2-amino-6-oxy-8 azapuraine (8-Azaguanine)-resistant BALB/c mouse myeloma-derived cultured cell line (P3-X63-Ag8・653, hereinafter abbreviated as X63 cells) was used as a parent cell line. The X63 cells were subcultured in RPMI-1640 culture medium (20 μg/ml, containing 8-Azaguanine) supplemented with 5% inactivated fetal calf serum (FCS), and X63 cells in the logarithmic growth phase were washed 3 times with RPMI-1640 culture medium and counted.

Cell fusion is performed in RPMI-1640 culture medium containing polyethylene glycol 4000 at a concentration of 50 (w/v)%.

That is, spleen cells and X63 cells are mixed at a ratio of 10:1 and centrifuged at 1500 rpm for 5 minutes. Supernatant is removed, and cell pellets are thoroughly suspended and subjected to cell fusion according to the method of Kohler and Milstein using polyethylene glycol. Thereafter, the spleen cells are suspended in an HAT selective medium (10% FCS-added RPMI-1640 culture medium containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) so that the spleen cells are contained at a concentration of 3.5×10^{6} cells/ml. Then, the cell suspension is dispensed into each well of 96 well microtest plate by 100 μl and cultured in a carbonic acid gas incubator (37°C, 95% humidity, 8% carbonic acid gas). On day 1 and day 2 after the initiation of culture, HAT medium is added by one drop to each well and by 2 drops on day 7 and day 9 after the initiation of incubation, which is followed by further culture.

⑤ Screening

From 10 days after the initiation of culture, clone cells emerge. For confirmation of antibody production, hybridoma culture supernatant is subjected to an antigen-antibody reaction test.

That is, 50 μl each from hybridoma culture supernatant and human PGIS antigen liquid is placed in a U-bottomed microtiter plate and thereto is added 50 μl of 20% suspension of Sepharose 4B bound with anti-mouse immunoglobulin antibody. The mixture is stirred at room

temperature for one hour and left standing for 10 minutes. After confirmation of complete sedimentation of anti-mouse immunoglobulin antibody-bound Sepharose 4B on the bottom of the well, 20 μl of the supernatant is taken and determined for concentration of residual human PGIS in the supernatant by PGIS ELISA system. When anti-human PGIS monoclonal antibody against human PGIS is present in the hybridoma culture supernatant, human PGIS and anti-human PGIS monoclonal antibody react and anti-mouse immunoglobulin antibody-bound Sepharose 4B sediment is formed as an antigen-antibody complex to decrease the concentration of residual human PGIS in the supernatant, thus proving the presence of anti-human PGIS monoclonal antibody.

Reference Example 1: RNA blot analysis

RNA blot hybridization analysis was made to examine the influence of several kinds of cytokines on the expression of HAEC-derived human PGIS mRNA.

The entire RNA (30 μg) derived from each HAEC which was incubated for 24 hours with several kinds of cytokines [IL-1α (1 ng/ml), IL-1β (1 ng/ml), IL-6 (2.5 ng/ml), TNF-α (5 ng/ml) and TNF-β (1 ng/ml)] was denatured with formamide, electrophoresed on 1% agar gel containing 1.5% formaldehyde, and transferred onto a nylon membrane. A probe [pHPGIS 135 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] was labeled with [α-^{32}P]dCTP by random priming method [Feinberg, A.P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13].

Then, hybridization was applied according to the method described in Biochem. Biophys. Res. Commun. 178, p 1479-1484 (1991). The membrane obtained was washed with 0.1×SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS at 60°C, air-dried and autoradiographed. The results are shown in Fig. 11. The main band of the HAEC-derived human PGIS mRNA was found at about 6 kb and three other minor bands were found (3.2, 2.5 and 1.7 kb). The test results revealed that the expression of human PGIS mRNA incubated for 24 hours

with IL-1α, IL-1β or IL-6 increased about 2-fold as compared with the control without cytokine treatment. Accordingly, increase in PGI_2 production caused by cytokine is considered to be attributable to the increased expression and production of PGIS which was achieved by cytokine. Thus, the treatment with cytokine is an extremely useful method for increasing PGIS expression to increase PGIS activity, which in turn accelerates PGI_2 production.

Reference Example 2: *In vivo* distribution of PGIS mRNA

RNA blot analysis was made to examine the distribution of PGIS mRNA expression in human body. Specifically, a filter was purchased from Clone-Tech on which poly $(A)^+$ RNA of various human tissues was electrophoresed and blotted. hPGIS135 was labeled with ^{32}P by the aforementioned method and subjected to Northern blot hybridization under the same conditions as above.

The results are shown in Fig. 12 and Fig. 13. The results confirm that PGIS mRNA was abundantly expressed widely in human tissues, particularly, in uterus, heart, skeletal muscle, lung and prostate and at significant levels, though slightly, in small intestine, kidney, liver and brain. These results coincide with the conventional reports of enzymatic activity and distribution in tissue of immunological response of PGIS, thus suggesting various biological roles assumed by PGIS besides the action in the vascular system. The 6 kb main, strong band and 3 weak bands as shown in Fig. 11 were observed in all tissues mentioned above, though relative thickness among the weak bands varied between tissues. Such various modes of presence of transcription products suggest possible different splicing of mRNA or the presence of an analogous gene (isozyme) as found in prostaglandin endoperoxidase.

Sequence Listing

Sequence No. : 1

Sequence length : 16

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

GGGACAAGGA CCACAT 16

Sequence No. : 2

Sequence length : 20

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

CAAAAGTCGC CTGTGGAAGC 20

Sequence No. : 3

Sequence length : 18

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

CACAGGCGAC TTTTGACA 18

Sequence No. : 4

Sequence length : 18

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

TGCCTGCATC TCCTCTGA 18

Sequence No. : 5

Sequence length : 19

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

GACTCGAGTC GACATCGATT TTTTTTTTTT TTTTT 35

Sequence No. : 6

Sequence length : 17

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

GACTCGAGTC GACATCG 17

Sequence No. : 7

Sequence length : 48

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Features : 36th, 37th, 41st, 42nd, 46th and 47th symbol N means inosine in each occurrence.

Sequence

CUACUACUAC UAGGCCACGC GTCGACTAGT ACGGGNNGGG NNGGGNNG 48

Sequence No. : 8

Sequence length : 183

Sequence type : nucleic acid

Strandedness : double

Topology : linear

Molecule type : cDNA

Original source :

Organism : human

Strain : λhPGIS141

Features :

Name/Key : peptide

Location : 1..183

Identification method : S

Sequence

GGG GAC AAG GAC CAC ATG TGC AGT GTC AAA AGT CGC CTG TGG AAG CTG 48

Gly Asp Lys Asp His Met Cys Ser Val Lys Ser Arg Leu Trp Lys Leu

5 10 15

```
CTA TCC CCA GCC AGG CTG GCC AGG CGG GCC CAC CGG AGC AAA TGG CTG    96
Leu Ser Pro Ala Arg Leu Ala Arg Arg Ala His Arg Ser Lys Trp Leu
             20                  25                  30
GAG AGT TAC CTG CTG CAC CTG GAG GAG ATG GGT GTG TCA GAG GAG ATG   144
Glu Ser Tyr Leu Leu His Leu Glu Glu Met Gly Val Ser Glu Glu Met
        35                  40                  45
CAG GCA CGG GCC CTG GTG CTG CAG CTG TGG GCC ACA CAG               183
Gln Ala Arg Ala Leu Val Leu Gln Leu Trp Ala Thr Gln
    50                  55                  60
```

Sequence No. : 9

Sequence length : 792

Sequence type : nucleic acid

Strandedness : double

Topology : linear

Molecule type : cDNA

Original source :

Organism : human

Strain : pHPGIS36

Features :

Name/Key : CDS

Location : 20..790

Identification method : E

Name/Key : mat peptide

Location : 20..790

Identification method : S

Sequence

```
CTACTACTAC TAGGCCACGC GTCGACTAGT ACGGGGGGGG GGGGGGGGGG GCAGCCCCGC  60
CAGCCCCGCC AGCCCCGCG ATG GCT TGG GCC GCG CTC CTC GGC CTC CTG       109
                         Met Ala Trp Ala Ala Leu Leu Gly Leu Leu
                                                 5                  10
GCC GCA CTG TTG CTG CTG CTG CTA CTG AGC CGC CGC CGC ACG CGG CGA    157
Ala Ala Leu Leu Leu Leu Leu Leu Leu Ser Arg Arg Arg Thr Arg Arg
                                 15                  20                  25
CCT GGT GAG CCT CCC CTG GAC CTG GGC AGC ATC CCC TGG TTG GGG TAT    205
Pro Gly Glu Pro Pro Leu Asp Leu Gly Ser Ile Pro Trp Leu Gly Tyr
                             30                  35                  40
GCC TTG GAC TTT GGA AAA GAT GCT GCC AGC TTC CTC ACG AGG ATG AAG    253
Ala Leu Asp Phe Gly Lys Asp Ala Ala Ser Phe Leu Thr Arg Met Lys
                         45                  50                  55
GAG AAG CAC GGT GAC ATC TTT ACT ATA CTG GTT GGG GGC AGG TAT GTC    301
Glu Lys His Gly Asp Ile Phe Thr Ile Leu Val Gly Gly Arg Tyr Val
                     60                  65                  70
ACC GTT CTC CTG GAC CCA CAC TCC TAC GAC GCG GTG GTG TGG GAG CCT    349
Thr Val Leu Leu Asp Pro His Ser Tyr Asp Ala Val Val Trp Glu Pro
                 75                  80                  85                  90
CGC ACC AGG CTC GAC TTC CAT GCC TAT GCC ATC TTC CTC ATG GAG AGG    397
Arg Thr Arg Leu Asp Phe His Ala Tyr Ala Ile Phe Leu Met Glu Arg
                                 95                 100                 105
ATT TTT GAT GTG CAG CTT CCA CAT TAC AGC CCC AGT GAT GAA AAG GCC    445
Ile Phe Asp Val Gln Leu Pro His Tyr Ser Pro Ser Asp Glu Lys Ala
                             110                 115                 120
AGG ATG AAA CTG ACT CTT CTC CAC AGA GAG CTC CAG GCA CTC ACA GAA    493
Arg Met Lys Leu Thr Leu Leu His Arg Glu Leu Gln Ala Leu Thr Glu
                         125                 130                 135
```

```
GCC ATG TAT ACC AAC CTC CAT GCA GTG CTG TTG GGC GAT GCT ACA GAA   541
Ala Met Tyr Thr Asn Leu His Ala Val Leu Leu Gly Asp Ala Thr Glu
    140                 145                 150
GCA GGC AGT GGC TGG CAC GAG ATG GGT CTC CTC GAC TTC TCC TAC AGC   589
Ala Gly Ser Gly Trp His Glu Met Gly Leu Leu Asp Phe Ser Tyr Ser
155                 160                 165                 170
TTC CTG CTC AGA GCC GGC TAC CTG ACT CTT TAC GGA ATT GAG GCG CTG   637
Phe Leu Leu Arg Ala Gly Tyr Leu Thr Leu Tyr Gly Ile Glu Ala Leu
                175                 180                 185
CCA CGC ACC CAT GAA AGC CAG GCC CAG GAC CGC GTC CAC TCA GCT GAT   685
Pro Arg Thr His Glu Ser Gln Ala Gln Asp Arg Val His Ser Ala Asp
            190                 195                 200
GTC TTC CAC ACC TTT CGC CAG CTC GAC CGG CTG CTC CCC AAA CTG GCC   733
Val Phe His Thr Phe Arg Gln Leu Asp Arg Leu Leu Pro Lys Leu Ala
        205                 210                 215
CGT GGC TCC CTG TCA GTG GGG GAC AAG GAC CAC ATG TGC AGT GTC AAA   781
Arg Gly Ser Leu Ser Val Gly Asp Lys Asp His Met Cys Ser Val Lys
    220                 225                 230
AGT CGC CTG TG                                                   792
Ser Arg Leu
235
```

Sequence No. : 10

Sequence length : 1296

Sequence type : nucleic acid

Strandedness : double

Topology : linear

Molecule type : cDNA

Original source :

Organism : human

Strain : pHPGIS135

Features :

Name/Key : CDS

Location : 3..827

Identification method : E

Name/Key : peptide

Location : 3..827

Identification method : S

Sequence

```
GG GAC AAG GAC CAC ATG TGC AGT GTC AAA AGT CGC CTG TGG AAG CTG      47
   Asp Lys Asp His Met Cys Ser Val Lys Ser Arg Leu Trp Lys Leu
                    5                  10                  15
CTA TCC CCA GCC AGG CTG GCC AGG CGG GCC CAC CGG AGC AAA TGG CTG    95
Leu Ser Pro Ala Arg Leu Ala Arg Arg Ala His Arg Ser Lys Trp Leu
                20                  25                  30
GAG AGT TAC CTG CTG CAC CTG GAG GAG ATG GGT GTG TCA GAG GAG ATG    143
Glu Ser Tyr Leu Leu His Leu Glu Glu Met Gly Val Ser Glu Glu Met
            35                  40                  45
CAG GCA CGG GCC CTG GTG CTG CAG CTG TGG GCC ACA CAG GGG AAT ATG    191
Gln Ala Arg Ala Leu Val Leu Gln Leu Trp Ala Thr Gln Gly Asn Met
        50                  55                  60
GGT CCC GCT GCC TTC TGG CTC CTG CTC TTC CTT CTC AAG AAT CCT GAA    239
Gly Pro Ala Ala Phe Trp Leu Leu Leu Phe Leu Leu Lys Asn Pro Glu
    65                  70                  75
```

```
GCC CTG GCT GCT GTC CGC GGA GAG CTC GAG AGT ATC CTT TGG CAA GCG     287
Ala Leu Ala Ala Val Arg Gly Glu Leu Glu Ser Ile Leu Trp Gln Ala
 80                  85                  90                  95
GAG CAG CCT GTC TCG CAG ACG ACC ACT CTC CCA CAG AAG GTT CTA GAC     335
Glu Gln Pro Val Ser Gln Thr Thr Thr Leu Pro Gln Lys Val Leu Asp
                100                 105                 110
AGC ACA CCT GTG CTT GAT AGC GTG CTG AGT GAG AGC CTC AGG CTT ACA     383
Ser Thr Pro Val Leu Asp Ser Val Leu Ser Glu Ser Leu Arg Leu Thr
            115                 120                 125
GCT GCC CCC TTC ATC ACC CGC GAG GTT GTG GTG GAC CTG GCC ATG CCC     431
Ala Ala Pro Phe Ile Thr Arg Glu Val Val Val Asp Leu Ala Met Pro
        130                 135                 140
ATG GCA GAC GGG AGA GAA TTC AAC CTG CGA CGT GGT GAC CGC CTC CTC     479
Met Ala Asp Gly Arg Glu Phe Asn Leu Arg Arg Gly Asp Arg Leu Leu
    145                 150                 155
CTC TTC CCC TTC CTG AGC CCC CAG AGA GAC CCA GAA ATC TAC ACA GAC     527
Leu Phe Pro Phe Leu Ser Pro Gln Arg Asp Pro Glu Ile Tyr Thr Asp
160                 165                 170                 175
CCA GAG GTA TTT AAA TAC AAC CGA TTC CTG AAC CCT GAC GGA TCA GAG     575
Pro Glu Val Phe Lys Tyr Asn Arg Phe Leu Asn Pro Asp Gly Ser Glu
                180                 185                 190
AAG AAA GAC TTT TAC AAG GAT GGG AAA CGG CTG AAG AAT TAC AAC ATG     623
Lys Lys Asp Phe Tyr Lys Asp Gly Lys Arg Leu Lys Asn Tyr Asn Met
            195                 200                 205
CCC TGG GGG GCG GGG CAC AAT CAC TGC CTG GGG AGG AGT TAT GCG GTC     671
Pro Trp Gly Ala Gly His Asn His Cys Leu Gly Arg Ser Tyr Ala Val
        210                 215                 220
```

```
AAC AGC ATC AAA CAA TTT GTG TTC CTT GTG CTG GTG CAC TTG GAC TTG    719
Asn Ser Ile Lys Gln Phe Val Phe Leu Val Leu Val His Leu Asp Leu
    225                 230                 235
GAG CTG ATC AAC GCA GAT GTG GAG ATC CCT GAG TTT GAC CTC AGC AGG    767
Glu Leu Ile Asn Ala Asp Val Glu Ile Pro Glu Phe Asp Leu Ser Arg
240                 245                 250                 255
TAC GGC TTC GGT CTG ATG CAG CCG GAA CAC GAC GTG CCC GTC CGC TAC    815
Tyr Gly Phe Gly Leu Met Gln Pro Glu His Asp Val Pro Val Arg Tyr
                260                 265                 270
CGC ATC CGC CCA TGACACAGGG AGCAGATGGA TCCACGTGCT CGCCTCTGCC        867
Arg Ile Arg Pro
            275
CAGCCTGCCC CAGCCTGCCC CAGCCTCCCA GCTTTCTGTG TGCACAGTTG GCCCGGGTGC  927
AGGTGCTAGC ATTACCACTT CCCTGCTTTT CTCCCAGAAG GCTGGGTCCA GGGGAGGGAA  987
AAGCTAAGAG GGTGAACAAA GAAAAGACAT TGAAAGCTCT ATGGATTATC CACTGCAAAG 1047
TTTTCTTTCC AAAATCAGGC TTTGTCTGCT CCCAATTCAC CTCGTTACTC TCACCTCGTG 1107
ATATCCACAA ATGCTATTCA GATAAGGCAG AACTAGGAGT CTTCACTGCT CTGCCCCCAA 1167
CTCCCGGAGG TGTCACCTTC CTAGTTCTTA TGAGCTAGCA TGGCCCGGGC CTTATCCAGT 1227
CAAAGCGGAT GCTGGCCACA GAAAGGCCAC TCAGGATGTC CTTTGTGTCC ATCGATGTCG 1287
ACTCGAGTC                                                          1296
```

Sequence No. : 11

Sequence length : 1977

Sequence type : nucleic acid

Strandedness : double

Topology : linear

Molecule type : cDNA

Original source :

Organism : human

Features :

Name/Key : CDS

Location : 28..1527

Identification method : E

Name/Key : mat peptide

Location : 28..1527

Identification method : S

Sequence

```
AGCCCCGCCA GCCCCGCCAG CCCCGCG ATG GCT TGG GCC GCG CTC CTC GGC        51
                               Met Ala Trp Ala Ala Leu Leu Gly
                                                 5
CTC CTG GCC GCA CTG TTG CTG CTG CTG CTA CTG AGC CGC CGC CGC ACG      99
Leu Leu Ala Ala Leu Leu Leu Leu Leu Leu Leu Ser Arg Arg Arg Thr
     10                  15                  20
CGG CGA CCT GGT GAG CCT CCC CTG GAC CTG GGC AGC ATC CCC TGG TTG     147
Arg Arg Pro Gly Glu Pro Pro Leu Asp Leu Gly Ser Ile Pro Trp Leu
 25                  30                  35                  40
GGG TAT GCC TTG GAC TTT GGA AAA GAT GCT GCC AGC TTC CTC ACG AGG     195
Gly Tyr Ala Leu Asp Phe Gly Lys Asp Ala Ala Ser Phe Leu Thr Arg
                 45                  50                  55
ATG AAG GAG AAG CAC GGT GAC ATC TTT ACT ATA CTG GTT GGG GGC AGG     243
Met Lys Glu Lys His Gly Asp Ile Phe Thr Ile Leu Val Gly Gly Arg
             60                  65                  70
TAT GTC ACC GTT CTC CTG GAC CCA CAC TCC TAC GAC GCG GTG GTG TGG     231
Tyr Val Thr Val Leu Leu Asp Pro His Ser Tyr Asp Ala Val Val Trp
         75                  80                  85
```

GAG CCT CGC ACC AGG CTC GAC TTC CAT GCC TAT GCC ATC TTC CTC ATG 339
Glu Pro Arg Thr Arg Leu Asp Phe His Ala Tyr Ala Ile Phe Leu Met
90 95 100

GAG AGG ATT TTT GAT GTG CAG CTT CCA CAT TAC AGC CCC AGT GAT GAA 387
Glu Arg Ile Phe Asp Val Gln Leu Pro His Tyr Ser Pro Ser Asp Glu
105 110 115 120

AAG GCC AGG ATG AAA CTG ACT CTT CTC CAC AGA GAG CTC CAG GCA CTC 435
Lys Ala Arg Met Lys Leu Thr Leu Leu His Arg Glu Leu Gln Ala Leu
125 130 135

ACA GAA GCC ATG TAT ACC AAC CTC CAT GCA GTG CTG TTG GGC GAT GCT 483
Thr Glu Ala Met Tyr Thr Asn Leu His Ala Val Leu Leu Gly Asp Ala
140 145 150

ACA GAA GCA GGC AGT GGC TGG CAC GAG ATG GGT CTC CTC GAC TTC TCC 531
Thr Glu Ala Gly Ser Gly Trp His Glu Met Gly Leu Leu Asp Phe Ser
155 160 165

TAC AGC TTC CTG CTC AGA GCC GGC TAC CTG ACT CTT TAC GGA ATT GAG 579
Tyr Ser Phe Leu Leu Arg Ala Gly Tyr Leu Thr Leu Tyr Gly Ile Glu
170 175 180

GCG CTG CCA CGC ACC CAT GAA AGC CAG GCC CAG GAC CGC GTC CAC TCA 627
Ala Leu Pro Arg Thr His Glu Ser Gln Ala Gln Asp Arg Val His Ser
185 190 195 200

GCT GAT GTC TTC CAC ACC TTT CGC CAG CTC GAC CGG CTG CTC CCC AAA 675
Ala Asp Val Phe His Thr Phe Arg Gln Leu Asp Arg Leu Leu Pro Lys
205 210 215

CTG GCC CGT GGC TCC CTG TCA GTG GGG GAC AAG GAC CAC ATG TGC AGT 723
Leu Ala Arg Gly Ser Leu Ser Val Gly Asp Lys Asp His Met Cys Ser
220 225 230

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GTC AAA AGT CGC CTG TGG AAG CTG CTA TCC CCA GCC AGG CTG GCC AGG     771
Val Lys Ser Arg Leu Trp Lys Leu Leu Ser Pro Ala Arg Leu Ala Arg
        235                 240                 245
CGG GCC CAC CGG AGC AAA TGG CTG GAG AGT TAC CTG CTG CAC CTG GAG     819
Arg Ala His Arg Ser Lys Trp Leu Glu Ser Tyr Leu Leu His Leu Glu
    250                 255                 260
GAG ATG GGT GTG TCA GAG GAG ATG CAG GCA CGG GCC CTG GTG CTG CAG     867
Glu Met Gly Val Ser Glu Glu Met Gln Ala Arg Ala Leu Val Leu Gln
265                 270                 275                 280
CTG TGG GCC ACA CAG GGG AAT ATG GGT CCC GCT GCC TTC TGG CTC CTG     915
Leu Trp Ala Thr Gln Gly Asn Met Gly Pro Ala Ala Phe Trp Leu Leu
                285                 290                 295
CTC TTC CTT CTC AAG AAT CCT GAA GCC CTG GCT GCT GTC CGC GGA GAG     963
Leu Phe Leu Leu Lys Asn Pro Glu Ala Leu Ala Ala Val Arg Gly Glu
            300                 305                 310
CTC GAG AGT ATC CTT TGG CAA GCG GAG CAG CCT GTC TCG CAG ACG ACC    1011
Leu Glu Ser Ile Leu Trp Gln Ala Glu Gln Pro Val Ser Gln Thr Thr
        315                 320                 325
ACT CTC CCA CAG AAG GTT CTA GAC AGC ACA CCT GTG CTT GAT AGC GTG    1059
Thr Leu Pro Gln Lys Val Leu Asp Ser Thr Pro Val Leu Asp Ser Val
    330                 335                 340
CTG AGT GAG AGC CTC AGG CTT ACA GCT GCC CCC TTC ATC ACC CGC GAG    1107
Leu Ser Glu Ser Leu Arg Leu Thr Ala Ala Pro Phe Ile Thr Arg Glu
345                 350                 355                 360
GTT GTG GTG GAC CTG GCC ATG CCC ATG GCA GAC GGG AGA GAA TTC AAC    1155
Val Val Val Asp Leu Ala Met Pro Met Ala Asp Gly Arg Glu Phe Asn
                365                 370                 375
```

CTG CGA CGT GGT GAC CGC CTC CTC CTC TTC CCC TTC CTG AGC CCC CAG 1203
Leu Arg Arg Gly Asp Arg Leu Leu Leu Phe Pro Phe Leu Ser Pro Gln
380 385 390
AGA GAC CCA GAA ATC TAC ACA GAC CCA GAG GTA TTT AAA TAC AAC CGA 1251
Arg Asp Pro Glu Ile Tyr Thr Asp Pro Glu Val Phe Lys Tyr Asn Arg
395 400 405
TTC CTG AAC CCT GAC GGA TCA GAG AAG AAA GAC TTT TAC AAG GAT GGG 1299
Phe Leu Asn Pro Asp Gly Ser Glu Lys Lys Asp Phe Tyr Lys Asp Gly
410 415 420
AAA CGG CTG AAG AAT TAC AAC ATG CCC TGG GGG GCG GGG CAC AAT CAC 1347
Lys Arg Leu Lys Asn Tyr Asn Met Pro Trp Gly Ala Gly His Asn His
425 430 435 440
TGC CTG GGG AGG AGT TAT GCG GTC AAC AGC ATC AAA CAA TTT GTG TTC 1395
Cys Leu Gly Arg Ser Tyr Ala Val Asn Ser Ile Lys Gln Phe Val Phe
445 450 455
CTT GTG CTG GTG CAC TTG GAC TTG GAG CTG ATC AAC GCA GAT GTG GAG 1443
Leu Val Leu Val His Leu Asp Leu Glu Leu Ile Asn Ala Asp Val Glu
460 465 470
ATC CCT GAG TTT GAC CTC AGC AGG TAC GGC TTC GGT CTG ATG CAG CCG 1491
Ile Pro Glu Phe Asp Leu Ser Arg Tyr Gly Phe Gly Leu Met Gln Pro
475 480 485
GAA CAC GAC GTG CCC GTC CGC TAC CGC ATC CGC CCA TGACACAGGG 1537
Glu His Asp Val Pro Val Arg Tyr Arg Ile Arg Pro
490 495 500
AGCAGATGGA TCCACGTGCT CGCCTCTGCC CAGCCTGCCC CAGCCTGCCC CAGCCTCCCA 1597
GCTTTCTGTG TGCACAGTTG GCCCGGGTGC AGGTGCTAGC ATTACCACTT CCCTGCTTTT 1657
CTCCCAGAAG GCTGGGTCCA GGGGAGGGAA AAGCTAAGAG GGTGAACAAA GAAAAGACAT 1717
TGAAAGCTCT ATGGATTATC CACTGCAAAG TTTTCTTTCC AAAATCAGGC TTTGTCTGCT 1777

CCCAATTCAC CTCGTTACTC TCACCTCGTG ATATCCACAA ATGCTATTCA GATAAGGCAG 1837

AACTAGGAGT CTTCACTGCT CTGCCCCCAA CTCCCGGAGG TGTCACCTTC CTAGTTCTTA 1897

TGAGCTAGCA TGGCCCGGGC CTTATCCAGT CAAAGCGGAT GCTGGCCACA GAAAGGCCAC 1957

TCAGGATGTC CTTTGTGTCC 1977

Sequence No. : 12

Sequence length : 500

Sequence type : amino acid

Topology : linear

Molecule type : peptide

Sequence

Met Ala Trp Ala Ala Leu Leu Gly Leu Leu Ala Ala Leu Leu Leu Leu
5 10 15

Leu Leu Leu Ser Arg Arg Arg Thr Arg Arg Pro Gly Glu Pro Pro Leu
20 25 30

Asp Leu Gly Ser Ile Pro Trp Leu Gly Tyr Ala Leu Asp Phe Gly Lys
35 40 45

Asp Ala Ala Ser Phe Leu Thr Arg Met Lys Glu Lys His Gly Asp Ile
50 55 60

Phe Thr Ile Leu Val Gly Gly Arg Tyr Val Thr Val Leu Leu Asp Pro
65 70 75 80

His Ser Tyr Asp Ala Val Val Trp Glu Pro Arg Thr Arg Leu Asp Phe
85 90 95

His Ala Tyr Ala Ile Phe Leu Met Glu Arg Ile Phe Asp Val Gln Leu
100 105 110

Pro His Tyr Ser Pro Ser Asp Glu Lys Ala Arg Met Lys Leu Thr Leu
115 120 125

Leu His Arg Glu Leu Gln Ala Leu Thr Glu Ala Met Tyr Thr Asn Leu
130 135 140
His Ala Val Leu Leu Gly Asp Ala Thr Glu Ala Gly Ser Gly Trp His
145 150 155 160
Glu Met Gly Leu Leu Asp Phe Ser Tyr Ser Phe Leu Leu Arg Ala Gly
165 170 175
Tyr Leu Thr Leu Tyr Gly Ile Glu Ala Leu Pro Arg Thr His Glu Ser
180 185 190
Gln Ala Gln Asp Arg Val His Ser Ala Asp Val Phe His Thr Phe Arg
195 200 205
Gln Leu Asp Arg Leu Leu Pro Lys Leu Ala Arg Gly Ser Leu Ser Val
210 215 220
Gly Asp Lys Asp His Met Cys Ser Val Lys Ser Arg Leu Trp Lys Leu
225 230 235 240
Leu Ser Pro Ala Arg Leu Ala Arg Arg Ala His Arg Ser Lys Trp Leu
245 250 255
Glu Ser Tyr Leu Leu His Leu Glu Glu Met Gly Val Ser Glu Glu Met
260 265 270
Gln Ala Arg Ala Leu Val Leu Gln Leu Trp Ala Thr Gln Gly Asn Met
275 280 285
Gly Pro Ala Ala Phe Trp Leu Leu Leu Phe Leu Leu Lys Asn Pro Glu
290 295 300
Ala Leu Ala Ala Val Arg Gly Glu Leu Glu Ser Ile Leu Trp Gln Ala
305 310 315 320
Glu Gln Pro Val Ser Gln Thr Thr Thr Leu Pro Gln Lys Val Leu Asp
325 330 335
Ser Thr Pro Val Leu Asp Ser Val Leu Ser Glu Ser Leu Arg Leu Thr
340 345 350

Ala Ala Pro Phe Ile Thr Arg Glu Val Val Val Asp Leu Ala Met Pro
355 360 365
Met Ala Asp Gly Arg Glu Phe Asn Leu Arg Arg Gly Asp Arg Leu Leu
370 375 380
Leu Phe Pro Phe Leu Ser Pro Gln Arg Asp Pro Glu Ile Tyr Thr Asp
385 390 395 400
Pro Glu Val Phe Lys Tyr Asn Arg Phe Leu Asn Pro Asp Gly Ser Glu
405 410 415
Lys Lys Asp Phe Tyr Lys Asp Gly Lys Arg Leu Lys Asn Tyr Asn Met
420 425 430
Pro Trp Gly Ala Gly His Asn His Cys Leu Gly Arg Ser Tyr Ala Val
435 440 445
Asn Ser Ile Lys Gln Phe Val Phe Leu Val Leu Val His Leu Asp Leu
450 455 460
Glu Leu Ile Asn Ala Asp Val Glu Ile Pro Glu Phe Asp Leu Ser Arg
465 470 475 480
Tyr Gly Phe Gly Leu Met Gln Pro Glu His Asp Val Pro Val Arg Tyr
485 490 495
Arg Ile Arg Pro
500

Sequence No. : 13
Sequence length : 24
Sequence type : nucleic acid
Strandedness : single
Topology : linear
Molecule type : other nucleic acid synthetic DNA
Sequence

GACAAGGACC ACATGTGCAG TGTC 24

Sequence No. : 14

Sequence length : 24

Sequence type : nucleic acid

Strandedness : single

Topology : linear

Molecule type : other nucleic acid synthetic DNA

Sequence

CTGTGTGGCC CACAGCTGCA GCAC 24